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Report

Twin Plants from Supernumerary Egg Cells in *Arabidopsis*

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Summary

Sexual reproduction of flowering plants is distinguished by double fertilization—the two sperm cells delivered by a pollen tube fuse with the two gametic cells of the female gametophyte, the egg and the central cell—inside the ovule to give rise to the embryo and the nutritive endosperm, respectively [1]. The pollen tube is attracted by nongametic synergid cells, and how these two cells of the female gametophyte are specified is currently unclear. Here, we show that *ALTERED MERISTEM PROGRAM 1* (*AMP1*), encoding a protein associated with the endoplasmic reticulum [2], is required for synergid cell fate during *Arabidopsis* female gametophyte development. Loss of *AMP1* function leads to supernumerary egg cells at the expense of synergids, enabling the generation of dizygotic twins. However, if twin embryos are formed, endosperm formation is prevented, eventually resulting in ovule abortion. The latter can be overcome by the delivery of supernumerary sperm cells in *tetraspore* (*tes*) pollen [3], enabling the formation of twin plants. Thus, both primary and supernumerary egg cells are fully functional in *amp1* mutant plants. Sporophytic *AMP1* expression is sufficient to prevent cell-fate change of synergids, indicating that one or more AMP1-dependent mobile signals from outside the female gametophyte can contribute to its patterning, in addition to the previously reported lateral inhibition between gametophytic cells [4–6]. Our results provide insight into the mechanism of synergid fate specification and emphasize the importance of specifying only one egg cell within the female gametophyte to ensure central-cell fertilization by the second sperm cell.

Results and Discussion

Several female-gametophytic defect mutants have been isolated from different genetic screens [7]. Most of these mutants, if not all, hardly proceed to successful double fertilization and are unable to produce viable fertilization products. Recent reports showed that loss-of-function mutations in several spliceosome factor genes, e.g., *LACHESIS* (*LIS*) or *CLOTHO/GAMETOPHYTE FACTOR 1* (*CLO/GFA1*), as well as in *WYRD* (*WYR*), which encodes a putative plant ortholog of the inner centromere protein (INCENP), lead to the ectopic expression of an egg cell marker in synergid cells [4, 5, 8]. It has been proposed that egg cell expression of *LIS* is required for synergid development [6]. However, the presumed additional egg cells in *lis*, *clo*, or *wyr* appear not to be functional. Synergids can also transdifferentiate to egg cell-like cells when the egg cell is ablated, and supernumerary egg cells have been proposed to be present in the *eostre* mutant of *Arabidopsis* likely due to the transdifferentiation of synergids [9, 10]. In the *eostre* mutant, in which *BEL1-LIKE HOMEODOMAIN 1* (*BLH1*) is misexpressed in the embryo sac, zygote-like structures were observed after pollination, but these structures did not give rise to embryos [10]. Several sporophytic defects have been reported for *amp1* mutants, including an enlarged shoot apical meristem, the early onset of flowering, and the overproliferation of suspensor cells, which occasionally leads to the formation of secondary embryos in later development [11–13]. To elucidate the details of secondary embryo formation in *amp1*, we examined ovules from *amp1-10* mutant plants from very early stages onward. Surprisingly, instead of suspensor-derived secondary embryos, which would be arranged in tandem as reported previously, we observed young twin embryos that were arranged side by side and thus appeared not to be suspensor derived (Figures 1A and 1B). To corroborate that loss of *AMP1* function is causal for this early twin-embryo phenotype, we analyzed two more *amp1* alleles: *amp1-13*, another T-DNA allele, and the ethyl-methanesulfonate-induced allele *amp1-1*, carrying a premature stop codon. Like *amp1-10*, *amp1-13* appears to be a null mutant (unpublished data; [13]). Indeed, the other two alleles showed the same twin-embryo phenotype in fertilized ovules (Figures 1C and 1D), although at somewhat different frequencies (Figure 1E). Because the early twin-embryo phenotype of *amp1-1* was rescued by two genomic *AMP1* constructs, *gAMP1* (zero twin-embryo pairs in 303 ovules) and *gAMP1:3xGFP* (zero twin-embryo pairs in 704 ovules), we concluded that lack of *AMP1* was causative for the early twin-embryo phenotype. However, ovules containing twin embryos aborted at early stages such that twin embryos did not develop beyond the early globular stage of embryogenesis (Figures S1A–S1C available online). This was likely linked to the fact that 95% (n = 111) of ovules containing twin embryos clearly lacked endosperm, which indicated that the supernumerary embryo was formed at the expense of central-cell fertilization. However, 19 of 265 fertilized ovules containing twin embryos showed autonomous central-cell divisions (Figures S1D–S1F).

To discern possible parental effects for the early twin-embryo phenotype, we performed reciprocal crosses between wild-type and *amp1-10* mutant plants. Although pollination of homozygous *amp1-10* plants with wild-type pollen resulted in twin embryos at a similar frequency as in the case of self-pollinated homozygous *amp1-10* mutant plants, no twin embryos were observed when wild-type plants were pollinated with *amp1-10* pollen (Figure 1E). To trace back this maternal defect, ovules of emasculated *amp1-10* flowers were analyzed. Often two or three cells with the nucleus at the egg cell position were observed, instead of only one as in wild-type embryo sacs (Figures 1F–1H). And in line with this result, the egg cell markers *pEC1.1::HTA6:3xGFP* and *gAT2G21740 (EC1.2):3xGFP* [14] were often expressed in two or even three cells in *amp1-10* mutant embryo sacs, whereas no supernumerary putative egg cells were observed in wild-type (Figures 2A–2E; Figures S2A–S2D). Because the total number of cells at the micropylar end of the ovule was not

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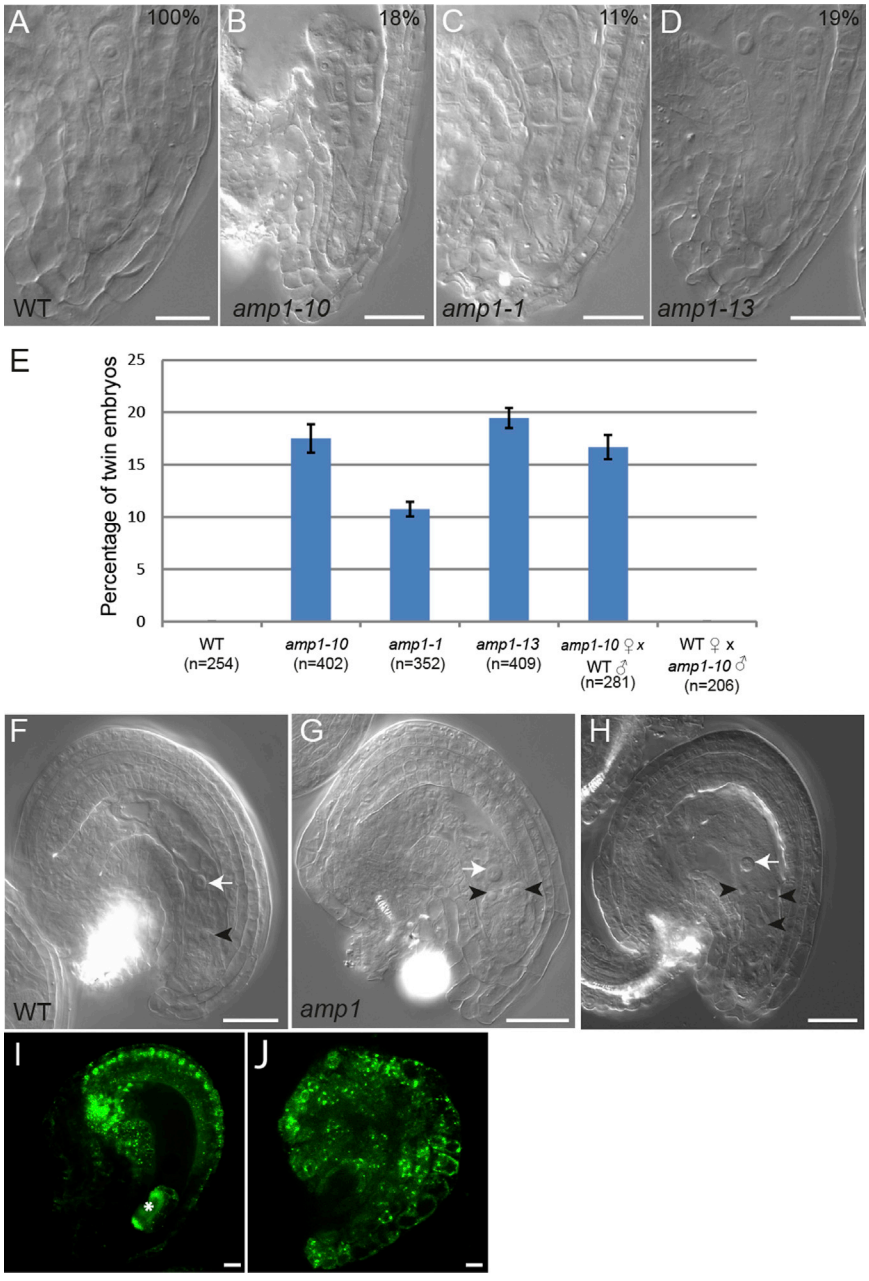

Figure 1. Twin Embryos and Supernumerary Putative Egg Cells in *amp1* Mutants

(A–E) Developing embryos. Single embryo in a wild-type (WT) ovule (A), twin embryos in *amp1-10* (B), *amp1-1* (C), and *amp1-13* (D) mutant ovules. (E) Frequency of twin embryos (expressed as percentage of fertilized ovules) in WT, *amp1*, and reciprocal crosses (mean ± SD). (F–H) Unfertilized ovules. One egg cell in WT (F); two or three putative egg cells in *amp1-10* (G and H). Black arrowhead, egg cell-like nucleus; white arrow, central-cell nucleus.

(I and J) *gAMP1:3GFP* expression in mature (I) and developing (J) ovule; asterisk, synergid.

Scale bars, 25 μm (A–H), 10 μm (I and J). See also Figure S1.

changed in *amp1* female gametophytes as compared to wild-type (Figures S1G–S1H″), the additional putative egg cell(s) must have been generated at the expense of some other cell(s). Because the synergids usually flank the single egg cell, they were prime candidates for such a fate substitution. Indeed, this idea was supported by the expression of the synergid marker *pNTA>>nTdtomato* (based on [15]). Although in wild-type almost always two cells expressed this marker and the fluorescently labeled nuclei were positioned like a synergid cell nucleus, five different categories were distinguished in *amp1-10*: (1) embryo sacs showed wild-type-like synergid marker expression; (2) two cells expressed the synergid marker, but in one cell the nucleus was shifted to an egg cell nucleus-like (ECL) position; (3) two cells expressed the synergid marker, and in both cells the nucleus was shifted to an ECL position; (4) only one cell expressed the synergid marker; and (5) no cell expressed the synergid marker (Figures 2F–2K; Figure S2E). These varied effects on synergid marker expression and nucleus position suggested that gametophytic cells destined to be synergids can adopt egg cell fate. To experimentally examine this idea, we analyzed *amp1-10* mutant ovules for expression of both the egg cell marker *pEC1.1::HTA6:3xGFP* and the synergid cell marker *pNTA>>nTdtomato* (Figures 2L and 2M). The vast majority of wild-type ovules contain two nuclei expressing only the synergid marker at the synergid cell nucleus position, in addition to one nucleus expressing the egg cell marker. In contrast, *amp1-10* mutant ovules displayed eight different categories of expression patterns and nuclear positions, with approximately 40% of these ovules harboring one or two nuclei at the synergid cell nucleus position that expressed both the egg cell and the synergid marker (categories II, III, IV, and VI; Figures 2L and 2M). Quantitative analysis of the single marker line *pEC1.1::HTA6:3xGFP* at earlier stages of female gametophyte development revealed that in 26% of all ovules that expressed the egg cell marker, there was at least one nucleus at the synergid cell nucleus position expressing that marker (Figures S2B and S2C). These results demonstrate that indeed the supernumerary putative egg cell(s) derive from transformed or misspecified synergids that might still retain the characteristic position of the synergid cell nucleus. Taken together, the above results indicated that *AMP1* is required to prevent synergids from taking on egg cell fate.

The persistent synergid marker *pNTA>>nTdtomato* was occasionally detected not only in nuclei at the egg cell position but—due to the stability of the fluorescent protein—also in one of the twin embryos (9.9%, n = 378 fertilized ovules) (Figures 3A–3C) in contrast to wild-type embryos (data not shown), clearly demonstrating that converted synergids when fertilized gave rise to embryos and were therefore fully functional egg cells. That supernumerary putative egg cells did not autonomously undergo embryo development without fertilization

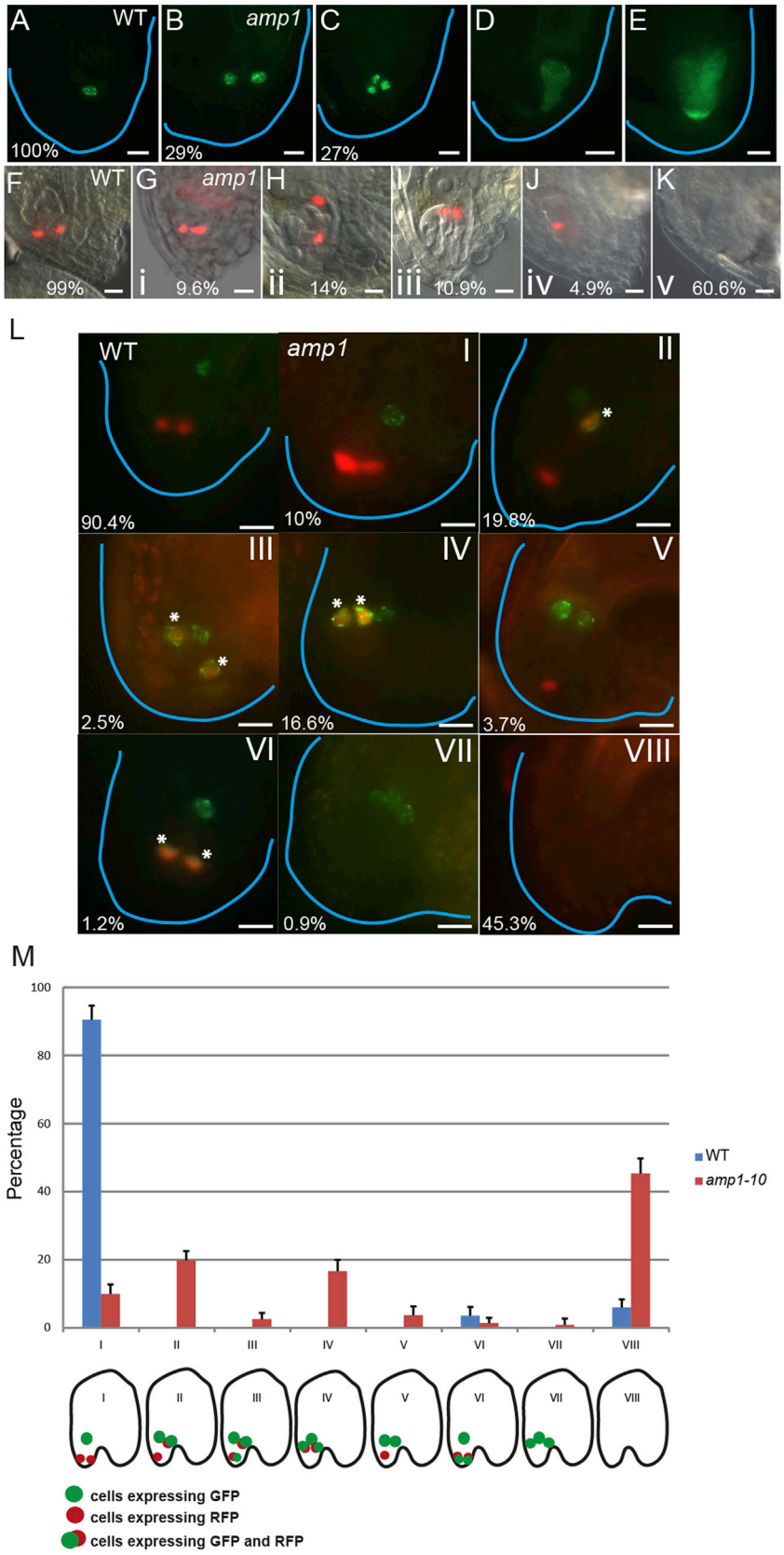

Figure 2. Synergids Expressing Egg Cell Marker in *amp1* Mutant Ovules

(A–E) Egg cell marker expression in wild-type (WT) and *amp1-10* (ovules outlined in blue). (A–C) *pEC1.1::HTA6:3GFP* in WT (A) and *amp1-10* (B and C); (D and E) *gAt2g21740(EC1.2):3GFP* in WT (D) and *amp1-10* (E).

(F–K) Synergid marker (*pNTA>>nTdtomato*) expression and nuclear position in WT (F) and *amp1-10* (G–K).

(L) Expression of *pEC1.1::HTA6:3GFP* and *pNTA>>nTdtomato* in WT and *amp1-10* (ovules outlined in blue). Asterisks, coexpression of both markers.

(M) Quantitative analysis of coexpression of egg cell marker and synergid marker (mean ± SD; n = 445 for WT and n = 480 for *amp1-10*); categories are the same as in (L).

Scale bars, 10 μm. See also Figure S2.

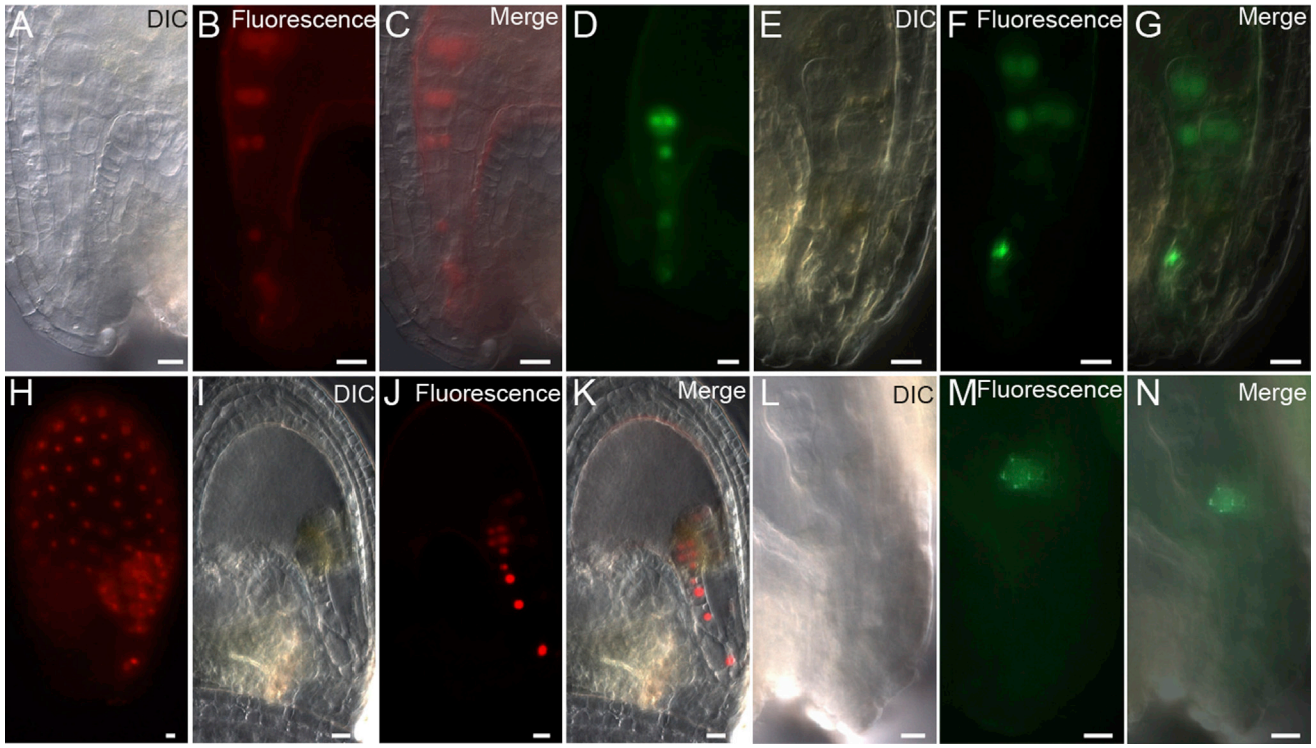

Figure 3. Supernumerary Putative Egg Cells Can Be Fertilized to Give Rise to Embryos

(A–C) Persistence of the synergid marker *pNTA>>nTdtomato* in one of the twin embryos in *amp1-10* ovule.

(D–G) Twin embryos are fertilization products. (D) Wild-type (WT) ♀ × *pS4::nGFP* ♂. (E–G) *amp1-10* ♀ × *pS4::nGFP* ♂.

(H–K) No endosperm formation in ovule containing twin embryos. (H) WT ♀ × *pRPS5A>>nTdtomato* ♂; (I–K) *amp1-10* ♀ × *pRPS5A>>nTdtomato* ♂.

(L–N) Egg cell marker *pEC1::HTA6:3GFP* in unfertilized egg cell next to nonfluorescent developing embryo in *amp1-10* ovule.

Scale bars, 10 μm. See also Figure S3.

was inferred from the observation that both embryos of the same twin pair in all GFP-expressing ovules (n = 36 twin pairs) expressed the paternally introduced early embryo marker *pS4::nGFP* (Figure S3A; Figures 3D–3G). To discern which *amp1* mutant ovules were preferentially fertilized, we analyzed ovules with GFP expression of the egg cell marker in wild-type and *amp1-10* (Figure S2D). Before fertilization, ovules displayed one, two, or three putative egg cells in roughly equal numbers. This distribution was changed after fertilization because the vast majority of *amp1-10* ovules containing three putative egg cells remained unfertilized, whereas the other categories of *amp1* ovules were preferentially fertilized (Figure S2D, compare left with right). These results were supported by the observation that only 50% of the *amp1-10* ovules were fertilized, and of those only about 20% (n = 623) contained twin embryos (Figure S2F). Thus, at least one cell with synergid properties appears to be required for successful fertilization.

Even though the above-mentioned lack of endosperm as well as the lack of central-cell fertilization in the case of early twin embryos (Figures 3H–3K; Figure S3B) already suggested that sperm cells from only a single pollen tube fused with female gametes in *amp1*, we performed a mixed-pollination experiment to distinguish sperm from different pollen tubes. A mixture of pollen carrying one or the other of the two embryo markers *pATML1::n3xGFP* and *pARF13>>nTdtomato* (Figure S3C) was used for pollinating *amp1-10* mutant plants. All the twin-embryo pairs examined (n = 22) expressed only one or the other of the two fluorescent markers (data not shown), which indicated that each twin-embryo pair originated from the pair of sperm delivered by a single pollen tube. Thus, embryo pairs in *amp1* mutants were genetically identical dizygotic twins.

Abortion of ovules with twin embryos should be overcome by delivering more than two sperm cells with a single pollen tube to achieve triple fertilization. This idea was based on the following observations: (1) central-cell marker expression was not changed in *amp1* (Figures S3D–S3G); (2) a supernumerary putative egg cell persisted in *amp1* fertilized ovules containing an embryo and endosperm (Figures 3L–3N: 16 of 125 ovules; Figures S3H and S3I: 12 of 130 ovules), which indicated no principal problem with central-cell fertilization in *amp1* embryo sacs containing two egg cells. Pollen of the *tetraspore* (*tes*) mutant often contain more than two sperm cells [3]. In contrast to self-pollinated *amp1* or pollination of *amp1* with wild-type pollen, pollination of *amp1* with *tes* pollen strongly decreased the percentage of endosperm absence in ovules containing twin embryos (Figure 4I). Moreover, pollination of *amp1* with *tes* pollen produced twin torpedo and bent-cotyledon stage embryos, which germinated as twin seedlings to give rise to twin adult plants (Figure 4).

Cell-cell communication has been proposed to play a central role for cell-fate specification in the *Arabidopsis* female gametophyte [4, 6]. We therefore investigated whether *AMP1* acts cell autonomously or rather non-cell-autonomously during synergid specification. The genomic *AMP1:3xGFP* fusion, which fully rescued the *amp1* supernumerary egg cell and twin-embryo phenotypes, was strongly expressed in the

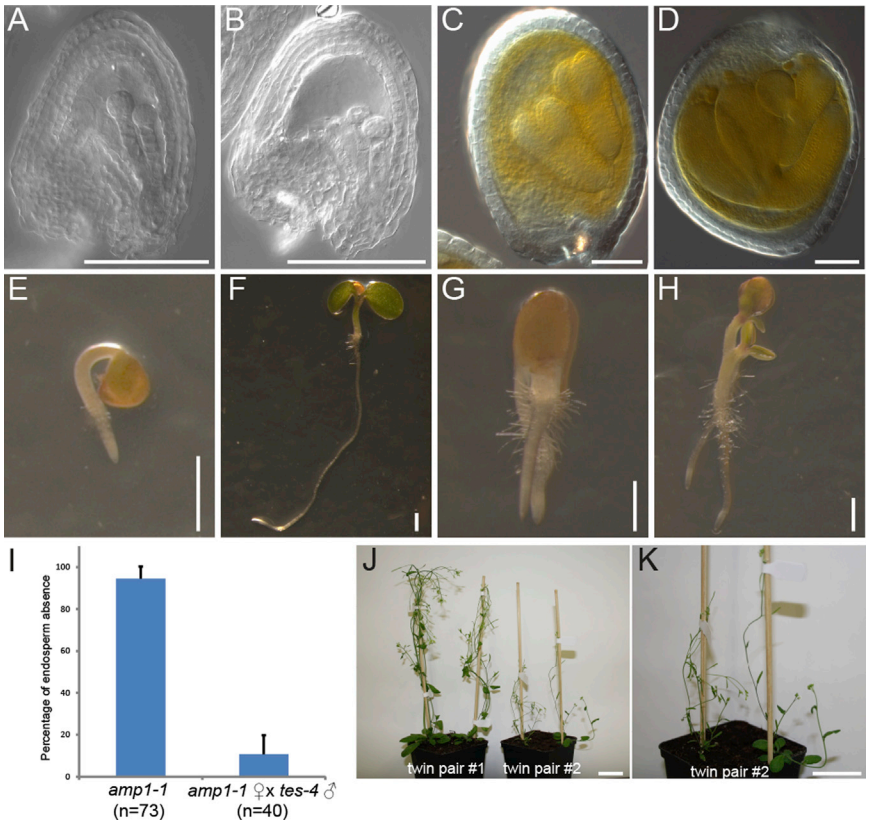

Figure 4. Twin Seedling and Plant Formation after Pollination of *amp1* Ovules with *tes-4* Pollen

(A–D) Cleared ovules of selfed *amp1* (A), *amp1-1* ♀ × Ws ♂ (B), and *amp1-1*♀ × *tes-4* ♂ (C and D). Scale bars, 0.1 mm.

(E–H) Germinated F1 seedlings from (E and F) *amp1-1* ♀ × Ws ♂. (G and H) *amp1-1* ♀ × *tes-4* ♂. Ws, Wassilewskija wild-type.

(I) Reduced frequency of endosperm absence by fertilization with supernumerary sperm. (n, total number of ovules containing twin embryos; mean ± SD).

(J and K) Adult twin plants. (J) Two independent twin pairs. (K) One twin of pair #2 appears weaker than the other.

Scale bars, 0.05 cm (E–H), 2 cm (J and K).

sporophytic tissue and the synergids, and weaker expression was sometimes detected in the egg cell (Figure 1I). *AMP1* expression at earlier stages of ovule development was only detected in the sporophytic tissue (Figure 1J). Given the strong *AMP1* expression in sporophytic tissue, we explored whether that expression contributed to proper synergid fate specification. Interestingly, *amp1* heterozygous plants only very rarely produced twin embryos (one case in 469 ovules) and supernumerary egg cells (one case in 121 ovules), indicating that sporophytic *AMP1* expression was principally able to mediate synergid fate specification. This finding was corroborated by the rescue of both mutant phenotypes in ten transgenic lines expressing *AMP1* from the *35S* promoter that is active in the surrounding sporophytic tissue but not in the female gametophyte itself ([16, 17]; Figures S3J and S3K; zero twin-embryo pairs in 358 ovules). Both phenotypes were also rescued in 19 transgenic lines expressing *AMP1* specifically in the synergids, using the *NTA* promoter ([15]; zero twin-embryo pairs in 132 ovules). Intriguingly, both mutant phenotypes could also be rescued by expressing *AMP1* in the neighboring central cell (24 transgenic lines, zero twin-embryo pairs in 351 ovules) and in the egg cell (25 transgenic lines, zero twin-embryo pairs in 426 ovules). Thus, synergid specification requires an AMP1-dependent signal that is likely mobile and can be provided by neighboring cells including the sporophytic tissue of the ovule.

There are distinct features that set *amp1* mutants apart from previously reported mutants with compromised synergid identity such as *lis* on which the lateral inhibition model for gametophytic cell-fate identity was based [4]. Unlike *lis*, *amp1* embryo sacs contained fully functional primary egg cell and supernumerary putative egg cell that gave rise to twin plants if supernumerary sperm were provided. Furthermore, *lis* is a gametophytic mutant, *LIS* was strongly expressed in egg cell and central cell, and egg cell expression was essential for synergid development [4, 6]. In contrast, the dizygotic twin and supernumerary egg cell phenotypes of *amp1* mutant can be rescued by sporophytic contribution of *AMP1* expression, which suggests that the AMP1-dependent signal for promoting or maintaining synergid cell fate can be provided by the gametophyte-surrounding maternal tissue. Thus, synergid fate might not simply be the result of preventing egg cell fate by lateral inhibition among the gametophytic cells at the micropylar end of the ovule, but the outcome of a distinct process also involving input from the surrounding sporophytic tissue. How AMP1 might contribute to the production of a synergid-promoting signal is not clear at present. AMP1 has been discussed to function as a glutamate carboxypeptidase, possibly influencing cytokinin levels or modulating levels of signaling molecules [18–20]. However, the expression of the sensitive synthetic cytokinin sensor *TCSn::GFP* [21] was not altered in *amp1* compared to wild-type ovules (Figures S3L and S3M). More recently, AMP1 has been localized to the ER and implicated in miRNA-mediated translational inhibition [2]. Whatever its exact molecular function, where in the ovule *AMP1* is expressed appears not to be critical, suggesting that AMP1 mRNA or protein might move between cells or be required for the production of a likely mobile signal for synergid identity.

Our analysis of the twin-embryo phenotype of *amp1* mutants also sheds light on the boundary conditions for double fertilization in plant reproduction, which involves two sperm cells and the four cells at the micropylar end of the female gametophyte: two synergids, one egg cell, and one central cell. Ovules with twin embryos but no endosperm as well as ovules with one developing embryo and endosperm plus one persisting unfertilized egg cell strongly suggest that the two sperm cells of a fertilizing pollen tube are free to choose their mating partners. This settles the controversial issue of potential mating preferences, which has largely been addressed by manipulating sperm cells [22–26] and a mutant in which specifically the central cell is not fertilized [27]. The occurrence of twin embryos without endosperm in *amp1* ovules also has implications regarding the number of synergids, which are required for pollen tube attraction [28]. Their number varies

between species [29]. One synergid is sufficient for pollen tube attraction such that any other synergid in the same ovule needs to be eliminated actively in order to prevent fertilization by another pollen tube [28, 30]. Our study now suggests that this rather cumbersome procedure might nonetheless have been selected for in evolution because the alternative—two egg cells and one synergid at the micropylar end of the ovule—reduces the probability of successful reproduction.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.11.021.

Author Contributions

J.K. conducted the experiments, and all three authors designed the study, analyzed the data, and wrote the manuscript.

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